A High Sensitivity Assay for Kinetic Analysis of Kinases and ATPases Based on Measurement of ADP Accumulation

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Abstract

DiscoveRx has developed a unique assay technology based on an enzyme-coupled detection system that measures ADP accumulation which has been widely applied for kinetic studies of proteins that utilize ATP, GTP or UTP. The kinetic assay format offers a number of benefits for kinase and ATPase research in particular, including a broad tolerance to additives, no need for a modified substrate, the ability to work with whole protein substrates, no requirement for a phospho-specific antibody, rapid signal generation, constant ATP concentration, and the ability to operate the assay on any simple fluorescence-based microplate reader. The kinetic capabilities of the assay are useful for assay development and enzyme characterization experiments, as well as more details analyses to determine compound mode of action, IC_{50}, and K_i values. Recently, DiscoveRx has made significant improvement in the technology to detect as little as 200 nM ADP in solution, while providing a S:B ratio greater than 3-fold for detection of 1-2 μM ADP, which is a commonly desired range for many screening assays. While most commonly used for kinase targets, the assay has also been applied to a range of other enzymes classes, such as ATPases. Furthermore, the assay can be used to measure other dinucleotides, and we will provide example data for measurement of UDP and GDP using this technology. In summary, the ADP detection technology offers a convenient and robust technology platform that can accelerate research programs based on its ease-of-use and simplicity for measuring enzyme kinetics.

ADP Assay Principle

Figure 1A: ADP Assay Principle

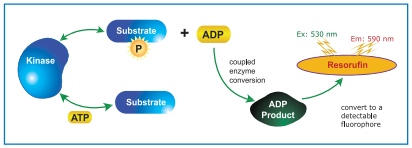
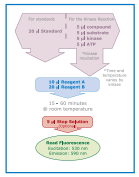


Figure 1B: ADP Assay Protocol



Format: Homogeneous

Plate: 96, 384, or 1536

Signal: Fluorescent Intensity

Reader: Standard microplate reader

Steps: 2-additions (Optional 3rd)

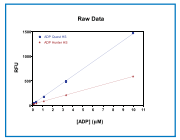
Incubation: 1 hour

The ADP HS Assays are a generic, non-antibody, non-radioactive assay for monitoring phosphotransferase (i.e. kinase and ATPase) activity. It is designed to be compatible with either peptide or whole-protein substrates in an endpoint assay format for screening. Unlike other generic methods, such as ATP depletion, this positive signal read-out assay detects the generation of ADP produced as a result of enzyme activity. The assays use an enzyme-coupled reaction and the fluorescent signal is red-shifted, minimizing interference from fluorescent compounds.

Methods

All kinase reactions and standard curves were performed in assay buffer containing 15 mM Hepes, 20 mM NaCl, 1 mM EGTA, 0.02 % Tween 20, 10 mM $MgCl_2$, 0.1% bovine gamma globulins, pH 7.4. Kinases were from Upstate, staurosporine from AG Scientific, substrates from American Peptide Co., and all other reagents obtained from Sigma-Aldrich. For β-(1-4) Galactosyltransferase assay the reaction was performed in the presence of 2 mM $MnCl_2$. Fluorescent intensity signal was measured using a Packard Fluorocount Reader using excitation/emission wavelengths of 530/590 nm with 0.1 s integration and PMT voltage of 750 V. For 1536 applications, reagents were dispensed using Deerac Equator dispenser and read on ViewLux reader.

ADP Quest and Hunter HS have improved S/B and sensitivity.



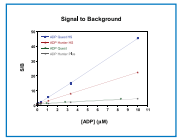
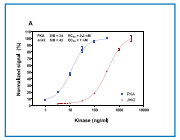


Figure 2. ADP Standard Curves. The ADP HS assays are designed to produce a positive signal in direct proportion to the amount of ADP produced. The assay was sensitive to 250 nM and linear to 10 μM ADP ($r^2 > 0.999$). At 1 μM ADP was 5.5 and 3.2 for Quest HS and Hunter HS respectively. This represents a 3 fold improvement in assay signal to background compared to ADP Quest and Hunter Plus.

Enhanced S/B and Sensitivity for Inhibitor Screens



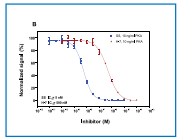


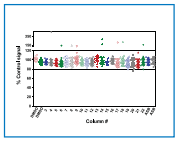
Figure 3. Performance in Kinase Assays. A. Kinases were incubated with kemptide (PKA) or ATF-2 (Jnk2α2) substrates and ATP. Maximal S/B of 34 and 42 were observed for PKA and Jnk2α2 respectively. **B.** IC_{50} values for PKA with staurosporine and H-7 are consistent with the literature and demonstrate the ability to identify weak inhibitors.

ADP Hunter HS: Designed for High Throughput Screening

	S:B		Sensitivity	Z' at 0.63μM ADP	% CV
	10 μM ADP	1 μM ADP			
ADP Hunter™ HS	21	3.3	160 nM	0.58	4.6
with DTT/Neutralization reagent	14	2.6	160 nM	0.51	4.2
with Stop solution	17	2.7	160 nM	0.48	4.3

Table 1. ADP Hunter HS Signal Stability. Performance of the Hunter HS has been enhanced when reducing agents such as DTT are used. Sensitivity and Z' are unaffected by the use of DTT in kinase reactions (Table 1). The ADP Hunter HS assay is specifically designed for batch reading of microplates, Signal stability is enhanced with the addition of the optional Stop Solution (Table 1). Hunter HS detection reagents act as a kinase reaction stop by inhibiting the activity of kinases, such as PKA. This provides an ideal endpoint readout and eliminates the need for EDTA.

ADP Hunter™ HS: Optimized for Inhibitor Screening

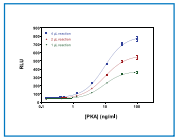


ADP Concentration	Cut Off Criteria	Number of Inhibitory Compounds	% of LOPAC Library
10 uM ADP	<80%	3	0.6%
1 uM ADP	<80%	5	0.8%

Figure 4 and Table 2. ADP Hunter HS LOPAC Library Screening. The ADP Hunter HS assay is specifically designed to minimize compound interference in high throughput screening. LOPAC compound interference was <0.8 % false positives at 1 μM ADP (Figure 4 and Table 2). Z' values of 0.88 and 0.66 were obtained for 10 and 1 μM ADP product respectively, indicating excellent suitability for high throughput screening.

ADP Hunter™ HS: Robust Assay Allows for Effortless Miniaturization

PKA kinase reaction using Hunter™ HS in 1536 format Raw Data



PKA enzyme reaction using Hunter™ HS in 1536 format Normalized Data

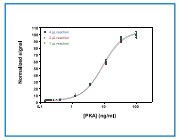
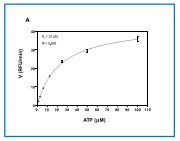


Figure 5. 1536-well Kinase Assay using ADP Hunter HS. Kinase assays were performed in 1536 assay plates using a Deerac Equator for dispensing. Reactions were performed in volumes down to 1 μL. Assay sensitivity was 100 nM ADP.

EC_{50} = 9 ng/mL for all assay volumes
Sensitivity < 0.14 ng/mL PKA
Max S/B = 19.5
% CV = 3.5 %
Max Z' = 0.9
Z' > 0.5 at 0.41 ng/mL
Identical performance even with 1 μL kinase reactions

ADP Quest™ HS: Characterization of kinase and inhibitor mode of action

Michealis-Menten plot for determining ATP K_m for PKA



Competitive Inhibition and K_i determination for PKA with staurosporine

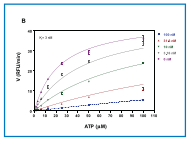
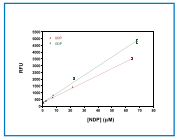


Figure 6. Characterization of kinase and inhibitor function using ADP Quest HS. **Figure 6A** demonstrates the use of the assay for determining the kinase K_m, which is important in target characterization. **Figure 6B** demonstrates the use in inhibitor K_i and mode of action determination, which is essential in hit characterization.

Assays can also detect UDP and GDP

ADP/UDP/GDP Standard Curve



UDP-Galactose in Galactosyltransferase assay Background Corrected Data

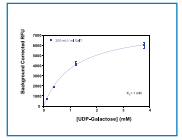


Figure 7. Detection of UDP and GDP with ADP Assays.

- Assays can also measure UDP and GTP
  - Similar dynamic range and sensitivity
- β-(1-4) Galactosyltransferase
  - Catalyzes formation of galactoside from UDP-galactose and acceptor sugar such as N-acetyl glucosamine (GlcNAc)
  - Titration of UDP-Galactose allows for substrate K_m to be determined

Summary

- **ADP Quest and Hunter HS are enhanced assay providing generic, non-radioactive detection of enzymes producing ADP**
  - High sensitivity and S/B with a positive linear signal readout
  - Suitable for kinases and ATPases with a wide range of ATP requirements
  - Works with unmodified peptide and whole protein substrates and good ATP tolerance with optional signal stop reagent
- **ADP Quest HS is ideal for kinase & inhibitor characterization**
  - Fast and accurate tool for determining K_m, K_i and mode of action
  - Kinetic or endpoint mode
- **ADP Hunter HS is designed for HTS**
  - Optimized for high throughput screening with minimal compound interference
  - Compatible with reducing agents such as DTT
  - Endpoint detection of ADP
- **All assays are suitable for detecting UDP and GDP producing enzyme**
  - Galactosyltransferases
  - GTPases
- **Broad instrument applicability**
- **Easily miniaturized**
  - No loss in performance in 1536 well format

DiscoveRx